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Environmental Studies of Schizophrenia Through the Prism of Epigenetics

Gabriel Oh[2] and Arturas Petronis[1,2]

[2]The Krembil Family Epigenetics Laboratory, Centre for Addiction and Mental Health, University of Toronto, 250 College Street, Toronto, Ontario M5T 1R8, Canada

Traditionally, etiological research of schizophrenia has been focused on elucidating predisposing genes and environmental risk factors. While numerous putative environmental hazards have been suggested, inconsistencies and methodological limitations of epidemiological studies have made it difficult to identify even a single exogenous cause of schizophrenia. Furthermore, there is increasing evidence that environmental risk factors may not play as much of a significant role in schizophrenia as previously suspected. In this article, we argue that molecular epigenetic studies can overcome the complexities of traditional epidemiological studies and may become a productive line of research in understanding the nongenetic mechanisms of schizophrenia.

Key words: schizophrenia/epidemiology/environment/epigenetics/DNA methylation

Introduction

It has been generally accepted that schizophrenia, like all other complex diseases, is caused by genetic and environmental factors. Traditionally, environmental contribution is measured by the degree of phenotypic differences in identical, or monozygotic (MZ), co-twins. Over the last 5 decades, numerous studies have been performed in order to identify specific environmental factors that would increase the risk for schizophrenia. Epidemiological studies revealed a long list of potential environmental risk factors, such as nonspecific stress, mental and physical abuse, maternal diet during pregnancy, drug use, living in an urban setting, migration, season of birth, and exposure to infections, among numerous other factors.[1–5] In addition to isolated environmental risk factors, there has been an increased interest in gene-environment (G × E) interactions, which assume nonadditive relationships between disease genes and hazardous environmental factors. While major effort has been made in the attempt to identify environmental risk factors of schizophrenia, inconsistencies and methodological limitations of epidemiological studies have hampered identification of causal exogenous factors for schizophrenia. In this article, we will briefly review the complexities of epidemiological studies of schizophrenia and suggest that molecular epigenetic strategies may enable one to bypass the limitations of the traditional environmental paradigms.

Methodological complexities in environmental studies of schizophrenia

Urbanicity and cannabis use are 2 of the more recently discovered and thoroughly investigated environmental risk factors for schizophrenia. There is overwhelming evidence that these 2 factors are linked to schizophrenia. In terms of urbanicity, it has been found that the incidence of schizophrenia is higher in urbanized areas as compared with rural areas. There are currently more than 10 studies that show this association after taking into account various confounding factors, such as age, sex, ethnicity, drug use, social class, and family history, among others.[6] In terms of cannabis use, a recent meta-analysis of 7 association studies consistently showed an increased risk for schizophrenia among the cannabis users.[7] While these studies are of significant interest and intuitively consistent with our general understanding of the origin of schizophrenia (urban living is more stressful than rural and active substances from cannabis may lead to neurochemical imbalances in the brain[8]), there are numerous complexities in the interpretation of the results of such epidemiological studies. First of all, it is very difficult to establish cause-effect relations between a putative environmental risk factor and the disease. Although exposure to environmental risk factors exhibits a strong association with the incidence of schizophrenia, the association is by no means a proof of causality. For example, the association between cannabis use and schizophrenia may be a result of self-medication to dull the already present psychotic symptoms, rather than a trigger for schizophrenia.[4] Furthermore, there is evidence that various environmental risk factors actually can be influenced by genetic factors.[9–13] For example, a 3372 twin pair–based study revealed that the concordance rate for cannabis use was 22.3% for MZ twins and 14.5% for dizygotic (DZ) twins ($P < .05$) (26.2% and

[1]To whom correspondence should be addressed; tel: 416-535-8501 ext 4880, fax: 416-979-4666, e-mail: arturas_petronis@camh.net.

16.5%, respectively, for illicit drugs in general, $P < .01$), which indicates an inherited predisposition to illicit drug use.[10] There are studies that also suggest that an individual who has an inherited risk for psychosis is more likely to use cannabis and also more likely to develop psychotic experiences when exposed to cannabis.[11–13]

In urbanicity studies, selective migration, where individuals with some specific (partially genetically determined) behavioral traits have tendencies to place themselves in hazardous environmental situations (in this case—large cities), can be one of the reasons behind its association with schizophrenia.[14] A recent study revealed that at least some of the urban-rural differences in schizophrenia risk were more likely to be of genetic, rather than environmental, origin.[15] Briefly, the authors looked at the association between the nearest older sibling's birthplace and the individual's risk for schizophrenia. If the nearest older sibling's birthplace has no effect on the individual's risk for schizophrenia, then urban-rural differences would be linked to the individual's own urban residence. However, if the nearest older sibling's birthplace has an independent effect, then some of the urban-rural differences would be linked to the family's urban residence prior to the individual's conception. In fact, the nearest older sibling's place of birth was independently associated with the risk for schizophrenia, even after taking into account for the individual's birthplace. Furthermore, the individual's place of birth (and upbringing) and the nearest older sibling's place of birth were virtually interchangeable in terms of schizophrenia risk, which suggests that some families may have a genetic liability that is related to the family's migration toward the city. This study showed that even if exposure to urban risk factors precedes the onset of schizophrenia, it may not play a causal role.

Twin studies revealed some further complexities in environment and genetic contributes to the choice of residential location.[16,17] The Australian twin study found significant effects of both shared environment and genes, where shared environment accounted for 50% of the variation in the youngest group but only about 10% in the oldest.[16,17] Interestingly, as contribution from shared environment decreased with age, genetic effects increased. The findings suggested that shared environment plays a more critical role for residential area selection among young individuals, while genes play a greater role in older individuals. However, a similar study conducted using data from The Netherlands twin register did not replicate the Australian findings. The study using Dutch twins found that there was no genetic contribution in selection for place of living in younger or older individuals but rather was entirely due to shared and nonshared environmental factors; the contribution from shared environment was greater among younger individuals, while nonshared environment played a greater role for older individuals.[17] The different outcomes from these 2 studies are thought to be due to the population characteristics of Australia, where there are less than 3 residents per square kilometer, as compared with The Netherlands, where there are more than 480 residents per square kilometer.[17] While it is relatively easy to move from one setting to another in Australia, this is not the case in The Netherlands. The different conclusions of the 2 studies once again illustrate the significant difficulties involved in isolating genetic and environment contribution.

More recently, there has been increasing interest in G × E interactions, and several interactions have been identified so far. For example, Caspi et al[18] found that a functional polymorphism in the catechol-O-methyltransferase gene moderated the influence of adolescent cannabis use on developing schizophrenia. Nicodemus et al[19] found significant G × E interactions between serious obstetric complications and polymorphisms in genes regulated by hypoxia or involved in the vascular function in the brain (*Protein kinase B* (*AKT1*), *brain-derived neurotrophic factor* (*BDNF*), *dystrobrevin binding protein 1* (*DTNBP1*), and *glutamate receptor - metabotropic 3* (*GRM3*)) for increased risk for schizophrenia. Other studies have found interactions between "familial liability" (rather than specific genes) and environmental risk factor. For example, van Os et al[20] found that there is a synergistic interaction between urbanicity and familial liability, where the effect of urbanicity was much larger for individuals with evidence of familial liability to schizophrenia as compared with those without. Although these G × E interaction findings are of significant interest, such data still need to be replicated. More importantly, the autonomy of environmental effects from genetics in the G × E studies, thus far, has not been proven. Therefore, it is difficult to draw any solid conclusions from these findings yet.

The role of environment becomes even more controversial in the light of some adoption and twin studies that do not reveal any evidence of environmental contributions to schizophrenia. For example, a study comparing a Finnish population of adopted children whose mothers had schizophrenia spectrum disorder with adoptees without genetic predisposition found that communication deviance in adoptive parents (ie, hostile family environment) does not increase the risk for schizophrenia unless the adoptee has a high genetic risk for schizophrenia.[21,22] Another study looked at the morbid risk for schizophrenia in the offspring of identical and nonidentical twins who were discordant for schizophrenia.[23] The risk for schizophrenia-like psychosis in the offspring of MZ twin was 16.8% for the affected twin and 17.4% for the normal co-twin, while the risk for DZ twin offspring was 17.4% and 2.1%, respectively, which suggests that genetic predisposition, rather than environmental influences, may play the critical role in schizophrenia. The latter observations are consistent with twin studies of normal (including behavioral) traits in twins, which compared phenotypic differences in MZ

twins who were reared together (MZT) with MZ twins who were reared apart (MZA).[24,25] Our current assumption of the effects of the environment on the phenotypic outcome dictates that MZA should be much more different than MZT due to the fact that they are raised in different environments. However, the test results revealed paradoxical findings. If the intraclass correlation (R) between MZA (R_{MZA}) and MZT (R_{MZT}) are expressed as a ratio (R_{MZA}/R_{MZT}), out of 22 measurements for which the R_{MZA}/R_{MZT} ratio was reported, 15 measurements had values over 0.9.[24,25] The 15 measurements include various scales of Multidimensional Personality Questionnaire, Raven Mill-Hill IQ Test, California Psychological Inventory, social attitudes on religious and nonreligious scales, electroencephalographic patterns, systolic blood pressure, heart rate, electrodermal response amplitude, and performance scale on the Wechsler Adult Intelligence Scale-IQ. The same ''anomaly'' was also detected in other MZ twin studies. For example, data gathered using the Swedish Twin Registry showed that for migraine susceptibility in female twins, the R_{MZA}/R_{MZT} ratio was 1.26.[26] Another study using the same twin registry showed that tobacco smoking in both males and females had a R_{MZA}/R_{MZT} of approximately 1.[27] The high R_{MZA}/R_{MZT} ratio suggests that environmental effects might not play as big of a role as previously suspected. Additional doubts about the role of environment on the phenotypic outcomes have also been found in animal studies. Inbred animals containing minimal genetic variation and cloned animals that technically should be genetically identical showed considerable phenotypic difference, even in the absence of detectable environmental variation.[28–31]

All the above-discussed complexities warrant a reevaluation of the environmental contribution to the etiopathogenesis of schizophrenia. Although the role of environmental factors in schizophrenia cannot be excluded, in the absence of good animal models of schizophrenia and in-depth knowledge of the degree of impact of gene-environmental correlation (whereby the genotype of an individual influences the exposure to specific environmental factors), it is nearly impossible to fully prove a causal association between a specific environmental hazard and risk for schizophrenia. We suggest that epigenetic studies of schizophrenia may shed a new light on our understanding of the putative environmental effects. The epigenetic paradigm of schizophrenia can shed a new light on the numerous yet unexplained findings in environmental studies of schizophrenia and estimate the putative environmental contributions in an empirically measurable manner, allowing the integration of inherited and acquired risk factors into a new theoretical framework.

A Primer to Epigenetics

Epigenetics by definition refers to the regulation of various genomic functions, including gene expression, which are not based on DNA sequence but rather controlled by heritable and potentially reversible chemical modifications of DNA and/or the chromatin structure.[32,33]

DNA methylation.

DNA methylation is a covalent modification of the DNA, and therefore, it is a relatively stable epigenetic mark. Cytosine methylation occurs at the 5' position of the pyrimidine ring and is catalyzed by several types of DNA methyltransferases (DNMTs). There are several DNMTs in the methyltransferase family, including DNMT1, DNMT3a, and DNMT3b. DNMT1 is thought to be the main enzyme responsible for the maintenance of DNA methylation. Several studies have shown that DNMT1 methylates hemimethylated DNA more rapidly than unmethylated DNA.[34,35] Therefore, despite the fact that DNMT1 have de novo methyltransferase activity,[36] it is believed that DNMT1 allows methylation profiles to be inherited from mother to daughter cell.[37] DNMTs require a methyl donor, *S*-adenosylmethionine (SAM), as a cofactor for cytosine methylation. SAM is a product of methionine metabolism, which involves multiple enzymes (betaine-homocysteine methyltransferase, methionine synthase, and methionine adenosyltransferase) and cofactors (vitamin B_{12}, betaine, folate, and homocysteine).[38] Cytosines in the CpG dinucleotide are the preferred, but not the exclusive, targets for DNA methylation.[39] Not all the CpG dinucleotides are methylated, but there is a cell-specific pattern of distribution of methylated CpG dinucleotides.[40] Most of the methylation occurs outside of the CpG islands, which are regions of high GC content (>55%) that colocalize with approximately 60% of all promoters.[41] Methylation of CpG islands is associated with gene regulation because the density of DNA methylation at such islands is often inversely proportional to the transcriptional activity of the gene.[42,43] DNA methylation patterns, like DNA sequences, are transmitted from maternal chromatids to daughter chromatids during mitosis, and this is called the epigenetic inheritance system.[44] In comparison to DNA sequences, the degree of mitotic fidelity of epigenetic patterns is approximately 3 orders of magnitude lower (10^{-6} and 10^{-3} for DNA sequences and DNA modification, respectively).[45] Such epigenetic metastability may result in significant epigenetic differences accumulated over time across the cells, despite their DNA sequence identity. It was commonly accepted that epigenetic patterns are erased in the early stages of germline cell development and that new patterns emerged after maturation.[37] However, there is increasing experimental evidence that some epigenetic signals do survive gametogenesis and that this information can be passed on from one generation to the next.[46–49] Therefore, epigenetic modifications are not only partially stable during mitosis but can also be transmitted transgenerationally via germline cells.

Chromatin modification

Histones are nuclear proteins, which are the basic building units of nucleosomes. A nucleosome consists of 147 base pairs of DNA wrapped around a protein octamer, made of pairs of 4 core histones, H2A, H2B, H3, and H4.[50,51] Each histone has a "tail" protruding out of the nucleosome, which can be modified in numerous ways: phosphorylated, ubiquitinated, sumoylated, acetylated, and methylated.[52] Histone methylation and acetylation at lysine residues on the histone tail have been the most thoroughly explored subtypes of histone modifications. Histone H3 dimethylation at lysine (K) 9 and trimethylation at K27 has been linked to the formation of transcriptionally inactive, condensed chromatin known as heterochromatin.[53] On the other hand, histone H3 and H4 acetylations on lysine residues and trimethylation of K4 (lysine) on H3 are generally associated with active gene transcription.[54,55] It has been proposed that histone acetylation modifies the chromatin structure in such a way that allows more open access thus allowing the binding of transcriptional complexes.[56]

Relationship Between DNA Methylation and Chromatin Modification

The mechanisms by which DNA methylation affects the regulation of gene activity are thought to be mediated in 2 ways. Firstly, methylated cytosines in transcription factor–binding sites change the affinity of DNA for the transcription factor, which in turn alters the transcriptional activity of a gene.[57,58] For example, DNA methylation at the promoter region of *BRCA1* exerts a suppressive effect on *BRCA1* expression by inhibiting cAMP response element-binding protein from binding to the promoter region.[59] Secondly, methylated cytosines attract methyl-CpG–binding protein, which recruit chromatin-remodeling proteins (ie, histone deacetylase [HDAC] complex and SWI-SNF proteins) to deacetylate the histones, resulting in transcriptional silencing.[60–65] Aberrant epigenetic regulation (epimutations) could have the same effect as DNA mutations because an epimutation could lead to the abnormal expression of a gene by enhancing or silencing that gene. Precise timing, location, and level of gene expression are crucial for normal cell function.

Epigenetic Insights on the Environmental Studies of Schizophrenia

Epigenetics may shed a new light on environmental studies of schizophrenia, and there are 2 aspects of epigenetics relevant to understanding of the nongenetic causes of this disease. Firstly, there is increasing experimental evidence that environmental agents alter the epigenetic status of specific genes and genomes. Therefore, epigenetic approaches can, in principle, empirically measure the effect of environment on a molecular level. The second aspect is related to the fundamental aspects of the environmental paradigm in schizophrenia. Epigenetics challenges one of the dogmas of current human morbid biology that discordance of identical twins is an indicator of environmental contribution to a disease.

Environmental Effects on Epigenetic Regulation

There is a growing body of evidence which suggests that DNA methylation may be modified by numerous environmental factors such as diet, drugs, and hormones and that epigenetic regulation of genes is a much more dynamic process than previously believed. Here are some examples of environmental factors, which may lead to changes in DNA methylation.

Diet. An example of diet affecting the phenotypic outcome is demonstrated by murine genes *Agouti* (A^{vy}) and *Axin* ($Axin^{Fu}$). The *Agouti* gene encodes a signaling peptide, which causes the melanocytes within hair follicles to change color from a spectrum of dark-brown-yellow.[67] The A^{vy} allele results from an intracisternal A particle (IAP) insertion upstream of the transcription starting site.[67,68] A cryptic promoter regulates the expression of the *Agouti* gene, which inversely correlates with CpG dinucleotide methylation of the A^{vy} IAP. Therefore, if the A^{vy} IAP is heavily methylated, the expression of the *Agouti* gene is turned off, leading to a dark fur color.[69] The *Axin* gene codes for the axin protein, which is involved in the mammalian embryonic axis formation.[70] $Axin^{Fu}$ contains an IAP insertion in the intron 6 of the gene.[71] This causes the expression of a truncated *Axin* gene, which originates from the IAP insert, as well as the expression of the wild type gene, which results in a double dose of Axin during development.[72] This results in varying degrees of kinks in the tail of $Axin^{Fu}$ mice. High methylation of the $Axin^{Fu}$ IAP causes the suppression of the variant Axin expression, leading to a normal phenotype.[73] Using A^{vy} and $Axin^{Fu}$ mouse models, studies have shown that an increased intake of maternal dietary methyl supplements during pregnancy, such as folic acid, vitamin B_{12}, and betaine, which increase DNA methylation, can result in different phenotypic outcomes. For example, increased maternal dietary methyl supplements caused the offspring to have dark fur in A^{vy} models.[68,74] Similar results were demonstrated in $Axin^{Fu}$ models, where maternal dietary methyl supplements during pregnancy led to nonkinked tail offspring.[75] Studies have also shown that the intake of folic acid affects both the global methylation level in the genome and the regulation of imprinted genes, such as *Igf2*, which have been implicated in a number of human cancers.[76–79] There is also evidence that maternal exposure to famine during pregnancy could increase the risk for schizophrenia in the progeny: children conceived at the height of

the Dutch Hunger Winter had 2-fold increase in risk for schizophrenia.[66] The increased risk for schizophrenia may be the result of epigenetic misregulation of genes triggered by malnutrition.

Drugs. Methamphetamine, which is known to cause schizophrenia-like phenotype with prolonged use, alters the expressional level of DNMT1.[80] In this connection, it is interesting to note that aberrant DNMT1 expression was also observed in the GABA (gamma-aminobutyric acid)-ergic interneurons of postmortem brain tissues of schizophrenia patients.[81] A recent study showed that histone acetylation is induced in the nucleus accumbens in response to acute and chronic cocaine administration.[82] The increase in histone acetylation was mediated by decreased HDAC function (more specifically, HDAC5). Interestingly, decreased HDAC5 function in the nucleus accumbens was also observed in chronic social defeat stress, an animal model for depression, suggesting that similar mechanisms may play a role in schizophrenia. Lastly, valproate, an anticonvulsant and mood stabilizer, is known to inhibit HDACs in vivo and in vitro.[83,84] Valproate is known to attenuate schizophrenia-like behavioral abnormalities in animal models.[85]

Stress. One of the first in vivo evidence of the impact of stress on epigenetic patterns came from animal studies. For example, it was recently discovered that exposure to nurturing behaviors (or the lack there of) alone could alter the epigenetic pattern. Pup licking, grooming, and arched-back nursing by mother rats induced histone modifications and changes in DNA methylation at the glucocorticoid receptor gene promoter in the hippocampus of the pups.[86] A recent animal study using contextual fear conditioning showed an increased transcription and demethylation of the *reelin* gene, 1 hour following fear conditioning.[87] The DNA methylation changes in this experiment may be caused by long-term potentiation–based learning, as well as a reaction to the stressful environment.

The value of studying environmental effects in epigenetics lies in the possibility of better understanding environmental risk factors and eliminating some of the confounding factors associated with epidemiological studies. The epigenetic approach to identifying the molecular effects of environmental factors might be a more productive line of research than direct, but methodologically limited, epidemiological studies. For example, it is relatively easy to identify epigenetic effects of cannabis on the brain using experimental animals or even humans by using postmortem brains or live cells such as peripheral blood, buccal epithelial cells, or neuronal cell lines. It is also feasible to perform an epigenetic comparison of individuals living in urban and rural places. Therefore, using epigenetics, it is possible to measure the true impact of the environment on an individual. Rather than focusing on whether the observed environmental risk factor is real, the question then becomes whether if and how the detected epigenetic changes in the unaffected individuals exposed to the putative risk factor, such as cannabis and urban life style, will be consistent with the epigenetic changes detected in schizophrenia patients compared with controls.[88]

Is Environment Really Important in Schizophrenia?

The epigenetic theory also challenges the idea that MZ twin discordance is an indicator of environmental effects. As previously mentioned, in comparison to DNA replication epigenetic patterns exhibit a substantially lower degree of stability, which to a large extent is stochastic rather than induced by specific environmental effects. This is well illustrated in the epigenetic studies of inbred animals.[47] Therefore, it is entirely possible that a substantial degree of stochastic epigenetic variation can be accumulated in MZ co-twins, and the resulting diverse phenotypic outcomes will be falsely interpreted as environmental contribution.

The idea of stochastic epigenetic change may explain some of the paradoxical findings in the MZA and MZT studies. Under the epigenetic theory, the finding that the differences between MZT and MZA are minimal for a large number of traits suggest that stochastic epigenetic changes may be a more important cause of phenotypic differences than environmental effects. It is possible that MZ twins are different for some traits not because they are exposed to differential environmental factors but rather because those traits are determined by metastable epigenetic regulation. The dogma that the nongenetic factors are environmental factors requires reevaluation.

Environmental Epigenetics of Schizophrenia: The Big Picture

Although the focus of this article has been on the epigenetic perspective of environmental risk factor, environmental epigenetics should not be analyzed separately from the other epigenetic aspects of schizophrenia. Epigenetics not only provides new insights for environmental studies, but epigenetic changes can also serve as a common etiopathological denominator for various epidemiological, clinical, and molecular findings of schizophrenia. Epigenetic mechanisms are consistent with the non-Mendelian mode of inheritance, as well as the presence of sporadic and familial cases of schizophrenia, sexual dimorphism, parental origin effect, late disease onset and coincidence with major hormonal changes in the organism, and fluctuating course of psychotic symptoms.[89–91] The epigenetic model of schizophrenia can be thought of as a result of a chain of deviant epigenetic events, which begins with a preepimutation (an epigenetic change that takes place during

gametogenesis or embryogenesis). A preepimutation increases the risk for schizophrenia, but it is not sufficient to cause the disease. The phenotypic outcome, ie, presence or absence of the disease, is dependent on the overall effect of a series of pre- and postnatal factors on the preepimutation. The epigenetic pattern is altered over time by the external environmental factors, stochastic events, and hormones, further increasing or decreasing the degree of the epigenetic misregulation. It may take decades for the epigenetic misregulation to reach a critical mass, beyond which the cell (or the tissue) is no longer functionally normal. Only a fraction of the predisposed individuals may reach the threshold of epigenetic misregulation that results in clinical symptoms of disease. The severity of epigenetic misregulation may fluctuate over time, which could lead to remission and relapse. In some cases, epimutations may slowly start regressing back to the normal state, which is seen as partial recovery.[92]

Until recently it was feasible to test only small genetic loci or limited regions and numbers of genes for epigenetic changes in disease. Today, technologies for high throughput, microarray-based epigenomic profiling, as well as reliable techniques have been developed, and such include both DNA methylation and histone modification studies.[93–95] It is even possible to perform a nonbiased epigenome-wide scan, which in the absence of good understanding of candidate loci, should be the ultimate goal of epigenomic studies of schizophrenia and other psychiatric and nonpsychiatric diseases. Fine mapping techniques, such as bisulfite modification coupled with various types of sequencing, allow us to look at specific genes with great resolutions. Therefore, we now have the experimental tools to test and characterize the extent to which epigenetic factors may change the traditional dyad of genes and environment.[96]

In epigenetic and epigenomic studies, there are some important confounding factors that must be considered when designing experiments or analyzing the data. Epigenotypes, unlike DNA sequences, are specific to various cell and tissue types. Therefore, tissues from the primary site of disease manifestations are needed for epigenetic analysis; in the case of psychiatric disorders, such tissue is the brain. Because the brain is made up of many different types of cells (various types of glial and neuronal cells) that may have differential epigenetic regulation, it is ideal to isolate specific cells from specific areas of the brain (ie, dopamine neurons from ventral tegmental area) using laser capture microdissection or flow cytometry. Furthermore, there is a need to identify common epigenetic markers between the affected tissue (ie, brain) and peripheral tissues (ie, peripheral lymphocyte) if it is to be used a diagnostics tool.

If epigenetic changes causal to schizophrenia are identified, the origin of such epimutations will not be immediately evident. Additional studies of germline and tissues not directly affected by the disease in both affected individuals and controls may help to differentiate inherited and acquired epimutations. The next task may be to differentiate between epigenetic changes induced by stochastic processes in the epigenetic machinery from the ones induced by some specific environmental effects. Based on the twin and inbred animal studies as well as the general feature of epigenetic metastability, our prediction is that the former will significantly outweigh the latter.

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